Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Titration of Herpesvirus of Turkeys (Strain FC-126) or Chicken Herpesvirus (Strain SB-1)

Date: October 29, 1998

Supersedes: June 1, 1984

Number: PYSAM0406.01

Standard Requirement: 9 CFR 113.330

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1. Introduction

This Supplemental Assay Method (SAM) describes a procedure using chick embryo fibroblast (CEF) cell cultures for titrating the herpesvirus of turkeys (Strain FC-126) or chicken herpesvirus strain SB-1 used as vaccines against Marek's disease. The vaccine is composed of a suspension of CEF cells infected with the virus.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Centrifuge (Beckman J-6B, JS-4.2 rotor)

2.1.2 Humidified, rotating egg incubator (Midwest Incubators, Model No. 252)

2.1.3 Water-jacketed incubator with a humidified 5% CO_2 atmosphere and temperature set at 37°C, (Forma Scientific, Model No. 3158)

2.1.4 Vortex mixer (Thermolyne Maxi Mix II Model No. M37615)

2.1.5 Magnetic stir plate

2.1.6 Scissors, sterile (Roboz Model No.RS-6800)

2.1.7 Curved tip forceps, sterile (V. Mueller SU 2315)

2.1.8 Microliter pipette (Rainin Pipetman, P1000, or equivalent)

2.1.9 250-ml trypsinizing flask with stir bar, sterile

2.2.10 Erlenmeyer flask with a stirring bar, sterile

2.1.11 Hemocytometer

2.1.12 Bunsen burner

2.1.13 Blunt thumb forceps, sterile

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 Cotton swabs

2.2.2 Tissue culture dish, 150x10 mm (Falcon, Cat. No. 1058)

2.2.3 Tissue culture dish, 100x20 mm (Falcon, Cat. No. 3003)

2.2.4 Plastic funnel covered with 4 layers of fine gauze

2.2.5 Polypropylene conical tube, 29x114 mm, sterile, 50 ml (Sarstedt, Cat. No. 62.547.205)

2.2.6 Polypropylene centrifuge tubes, 250 ml (Corning, Cat. No. 25350)

2.2.7 Roller bottles, 1000 ml (Falcon, Cat. No. 3007)

2.2.8 Serological pipets (Falcon, Cat. No. 7530)

2.2.9 60-mm gridded cell culture dish, tissue culture treated (Costar, Cat. No. 3160)

2.2.10 2 dozen specific-pathogen-free (SPF) chick embryos, 9- to 11-day-old

2.2.11 Fetal Bovine Serum (FBS)

2.2.12 L-Glutamine (Sigma, Cat. No. G7513)

2.2.13 Trypsin, 0.25% (Cello Corporation, Cat. No. AT25)

2.2.14 Pipette tips (Rainin 0-100, 0-200, 100-1000 or equivalent)

2.2.15 Solutions

All solutions are filter sterilized.

1. Trypsin Solution (0.25%):

NaCl	8.0 g
KCl	0.4 g
Glucose	1.0 ml
Phenol Red (0.5% solution)	1.0 ml
Trypsin (1:250)	2.5 g
$NaHCO_3$	0.35 g
q.s. with distilled or deionized water	1 L

Adjust pH to 7.4 with $NaHCO_3$ solution.

2. Growth Medium

Medium 199 (with Earle's salts)(powdered)	10 g
Nutrient Mixture F10 (powdered)	10 g
Bacto Tryptose Phosphate Broth (dry Powder)	2.95 g
$NaHCO_3$	2.5 g
Penicillin (potassium G)	200,000 units
Streptomycin	200 mg
Fetal Bovine Serum* (heat inactivated)	85 ml
q.s. with distilled or deionized water	2185 ml

Adjust pH to 7.35 to 7.4 by adding $NaHCO_3$ solution.
Before use, add 1.0 ml of a 200-mM concentration of L-glutamine per 100 ml medium.

3. Maintenance Medium

Medium 199 (with Earle's salts)(powdered)	10 g
Nutrient Mixture F10 (powdered)	10 g
Bacto Tryptose Phosphate Broth (dry powder)	2.95 g
$NaHCO_3$	2.75 g
Penicillin (potassium G)	200,000 units
Streptomycin	200 mg
Fetal Bovine Serum* (heat inactivated)	42 ml
q.s. with distilled or deionized water	2142 ml

Adjust pH to 7.5 by adding $NaHCO_3$ solution. Before use, add 1.0 ml of a 200-mM concentration of L-glutamine per 100 ml medium.

2.2.16 Cell cultures (Secondary CEF cultures are used for the titration.)

1. Preparing primary cultures

Prepare primary CEF cell cultures from 9- to 11-day-old embryos (derived from specific-pathogen-free flocks) in the following manner: Swab the air cell end of the egg with 70% ethanol, flame, and break open the shell with sterile blunt thumb forceps. Use the forceps to open the membranes, lift out the embryo, and place it in a sterile disposable petri dish. Four to six embryos may be prepared together. Remove (and discard) the heads of the embryos with sterile scissors. Wash the embryos by adding 0.25% trypsin solution to the petri dish. Open the body cavity of the embryos with the sterile forceps and remove the liver and the bulk of the other viscera. Gently squeeze the remainder of the embryos with the forceps to remove as much blood as possible. Pick the washed embryos out of the

*Previously tested for freedom from extraneous agents.

wash solution with the forceps, drain them momentarily, and place them in a sterile dry petri dish. Mince the embryos thoroughly by cutting with a sharp sterile scissors.

Place the minced tissue in a 250-ml sterile trypsinizing flask with a magnetic stirring bar, add 30 ml of 0.25% trypsin solution (prewarmed to approximately 35°C), and trypsinize for 20 min at room temperature. Carefully decant the supernatant suspension through a sterile funnel with 4 layers of gauze into a sterile centrifuge bottle.

Add approximately 30 ml of growth medium to the centrifuge bottle to stop the action of the trypsin on the cells. To the remaining fragments in the trypsinizing flask, add another 30 ml of 0.25% trypsin and repeat the process for another 20 min. Add this cell suspension to the first collection. Centrifuge at approximately 250 x g for 10 min. Observe the volume of packed cells, then remove the supernatant. Dilute the cells approximately 1:300 with growth medium and plant in suitable culture vessels. Incubate 3-5 days at 37°-37.5°C in a high humidity atmosphere containing approximately 5% CO_2. At this time, the cell monolayers should be confluent.

2. Preparing secondary cultures

Remove the medium from the primary culture vessel and add an appropriate volume of 0.25% trypsin solution to each vessel. Let the trypsin solution remain in contact with the cell sheet for 60-90 sec, then remove it.

Place the vessels in a horizontal position with the cell sheet down and incubate at 37°-37.5°C for an additional 10 to 20 min, or until the cell sheet appears to be well separated. The proper length of time will be learned by experience--too short a time will result in large clumps of cells in the new suspension. To each vessel, add an appropriate amount of fresh growth medium and shake or pipette to loosen and break up the cell clumps. Pour the cell suspensions into an Erlenmeyer flask with a stirring bar. After thorough mixing, make a cell count with a hemocytometer. Adjust the volume so that the cell

concentration is approximately 375,000 per ml. Plant the secondary cell suspension into 60-mm tissue culture dishes (gridded plastic dishes or plain dishes if a grid-adapted stage is to be used in microscopic observation for counting). Add 4 ml cell suspension per plate (approximately 1.5 million cells). Incubate the cultures at 37°-37.5°C in a high humidity atmosphere containing approximately 5% CO_2. When the cultures have reached confluency (24 hr or less), they are ready for inoculation (virus titration).

3. Preparation for the test

3.1 Personnel qualifications/training

The executor must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling and disposal of biological agents, reagents, tissue culture samples, and chemicals. The executor must also have knowledge of safe operating procedures and policies and Quality Assurance (QA) guidelines of the Center for Veterinary Biologics-Laboratory (CVB-L) or equivalent; and training in the operation of the necessary laboratory equipment listed in part **2.1**.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturer’s instructions and monitor in compliance with current corresponding CVB-L/National Veterinary Services Laboratories Standard Operating Procedures (SOPs) or equivalent.

3.3 Preparation of reagents/control procedures

Prepare reference viruses in the same manner as sample preparation.

3.4 Preparation of the sample

3.4.1 Remove 1 ampule of vaccine from the ultra-cold storage container and thaw quickly by immersing in a water bath approximately 25°C. Add the concentrated vaccine immediately to the appropriate amount (according to the indicated dosage) of the manufacturer's diluent (at room temperature). Do this by withdrawing the vaccine into a 10 ml syringe through an 18 gauge (or larger) needle, then withdraw approximately 5 ml of the diluent into the same syringe and mix gently. Slowly force the contents of the syringe into the bottle of diluent keeping the end of the needle in the liquid by tipping the bottle. Withdraw 2 ml from the diluted vaccine, use to rinse the ampule once, then add this back to the diluted vaccine. Withdrawal and expulsion must be done slowly to prevent rupturing of the cells. This mixture constitutes "field strength" vaccine.

3.4.2 Place the vaccine bottle in an ice bath for 2 hr (gently mix every 30 min) prior to proceeding with the titration. Shortly before the end of the 2 hr holding period, place 8.0 ml of growth medium (at 4°C) in 2 sterile test tubes and 9.0 ml of growth medium in 1 sterile test tube (make a set of 3 tubes for each vaccine sample). Use these to make further dilutions in the titration procedure. These dilution blanks are not held in an ice bath.

4. Performance of the test

4.1 Preparing dilutions and inoculating plates

Mix the vaccine by inverting the bottle 10 to 15 times. Withdraw a sample using a 2.5 ml syringe fitted with an 18 gauge needle and add 2.0 ml to the first tube with 8 ml diluent (1:5). Mix with a sterile 10 ml pipette and transfer 2.0 ml (of the 1:5) to a second tube with 8 ml diluent (1:25). Use a clean sterile 10 ml pipette; mix the 1:25 dilution; then transfer 1 ml (of the 1:25) to the third tube with 9 ml diluent (1:250). With SB-l virus, the third tube may contain 8 ml diluent, in which case 2 ml of vaccine is added, making a final dilution, of 1:125. Use a clean sterile pipette, mix the final dilution and inoculate 1.0 ml per plate into 5 test plates. Do this by drawing a large sample into a 10 ml pipette and distribute the inoculum into the 5 plates, using the graduations between 2 and 7 ml (this inoculum is in addition to the 4 ml medium already in each plate). Mixing must be done thoroughly but gently to prevent rupturing of the cells. Do this procedure of dilution and inoculation as rapidly as possible to prevent cells from attaching to the surface of the dilution tubes (less than 2 min should elapse between addition of the cell suspension to a particular dilution blank and the removal of a sample for further dilution or inoculation). Swirl the test plates as soon as each vaccine sample has been inoculated. Incubate the plates at 37°-37.5°C in a high humidity atmosphere containing 5% CO_2. Twenty-four hr postinoculation (PI), remove the medium from the plates and replace with 5 ml maintenance medium. The maintenance medium may be replaced after 2 or 3 days if the pH of the culture fluids becomes too acidic.

5. Interpretation of the test results

5.1 Controls

Titrate a known positive reference virus with each group of titrations. The titer of the positive reference must be within the established range for the test results to be valid. Uninoculated negative control cells are maintained to monitor the integrity of the cell culture system.

5.2 Making foci counts and calculating titer

5.2.1 Counting

Incubate the plates at 37°-37.5°C until the time for counting foci. With strain FC126, count the foci 5 days PI. With strain SB-l, count the foci 7 days PI. Use an inverted microscope (and a grid-adapted stage if plain plates have been used) to make the counts. Count all the foci on each of the plates of the titration series. A focus is counted as 1 regardless of size unless it has apparently arisen from 2 distinct centers.

5.2.2 Calculating

Calculate the average number of foci per plate and multiply this value by 25* or 50** depending on the dilution used in inoculation; this result will be the focus-forming units (FFUs) per bird dose (assuming the volume of 1 bird dose is 0.2 ml).

5.3 Retests

Conduct retests as required by The Code of Federal Redgulations, Title 9, Part 113.8 (b) and requirements of minimum release in firm’s current Outline of Production, Part V.

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*125 (dilution factor) divided by 5 (number of doses per ml) equals 25.
**250 (dilution factor) divided by 5 (number of doses per ml) equals 50.

5.4 Evaluation of test results

5.4.1 The 9 CFR 113.8(b) defines the criteria for a satisfactory/unsatisfactory serial.

5.4.2 The firm's requirements of minimum release/stability titers for each Marek's vaccine are listed in the current Outline of Production, Part V, for the specific product code.

6. Report of test results

Titers are reported out as FFUs per bird dose.

7. References

This document was rewritten to meet the current CVB-L QA SAM format. No significant changes were made from the previous protocol. This document supersedes the June 1, 1984, version.